Exhibit A

Measurement of the antinociceptive effects of MrIA in a rat model of neuropathic pain
Methods

Animals

Adult male Sprague-Dawley rats were purchased from the Animal Resources Centre (ARC), Perth, Australia, and the Herston Medical Research Centre, The University of Queensland. Rats were housed in a temperature controlled environment (21 ± 2° C) with a 12h/12h light/dark cycle. Food and water were available *ad libitum*. Ethical approval for 20 this study was obtained from the Animal Experimentation Ethics Committee of The University of Queensland.

Reagents and materials

Isoflurane (Forthane•) was obtained from Abbott Australasia Pty Ltd (Sydney, Australia). Sodium benzylpenicillin vials (600 mg) were purchased from CSL Ltd (Melbourne, Australia). Normal saline ampoules were obtained from Delta West Pty Ltd (Perth, Australia) and heparinised saline (50 IU/5 ml) was purchased from Astra Pharmaceuticals Pty Ltd (Sydney, Australia). Single lumen polyethylene tubing (I.D. 0.2 mm, O.D. 0.6 mm) was purchased from Auburn Plastics and Engineering Pty Ltd (Sydney, Australia). Sterile siliconized silk sutures (Dysilk□) were obtained from Dynek Pty Ltd (Adelaide, South Australia) and Michel clips were purchased from Medical and Surgical Requisites Pty Ltd (Brisbane, Australia).

Chronic Constriction Injury (CCI) of the Sciatic Nerve

Rats were anaesthetised with ketamine (80 mg/kg) and xylazine (8 mg/kg) administered by intraperitoneal injection, and a chronic constriction injury (CCI) of the sciatic nerve was produced according to the method of Bennett and Xie (1988). Briefly, the left common 15 sciatic nerve was exposed at mid-thigh level by blunt dissection through the biceps femoris. Proximal to the trifurcation, ≈ 10 mm of nerve was freed of adhering tissue and four loose ligatures (3.0 silk) were tied around the sciatic nerve (≈ 1 mm apart). The incision was closed in layers. After surgery, rats received benzylpenicillin (60 mg s.c.) to prevent infection and were kept warm during surgical recovery. Rats were housed singly 20 for 14 days prior to opioid or vehicle administration. Rats were inspected daily from the time of CCI-surgery with regard to posture of the affected hindpaw, exploring behaviour, body weight and water intake, and any signs of autotomy. Early signs of autotomy were seen in one rat (gnawing of claw tips and some surrounding tissue on the ipsalteral hindpaw) and this animal was promptly euthanased. 25

Intrathecal Catheter Insertion

Ten to eleven days post CCI-surgery or in untreated controls, rats were deeply anaesthetised with a mixture of ketamine (80 mg kg-1) and xylazine (8 mg/kg) administered as a single intraperitoneal (i.p.) injection. Prior to surgery, the back and neck 30 regions of the rat were shaved and the skin cleansed with betadine surgical scrub. The rat was then placed in a prone position and the L6 lumbar vertebra was located by palpation of the tuber sacrales of the os ileum (Hebel & Stromberg 1976). A 6 cm incision was made in the midline of the back, 3 cm caudal and 3 cm cephalad to L6. A subcutaneous pocket (for the intrathecal catheter) was formed by blunt dissection with scissors on both sides of the incision. The fascia covering the superficial shaped incision that encompassed L5. Additional 5 mm caudal incisions were made parallel to L6. The fascia was then retracted and the lumbar muscles surrounding the base of L5 and L6 were removed, as was the m. interspinalis between the spinous processes of L5-L6.

Following removal of the L6 spinous processes with rongeurs, the soft tissue beneath the L5 iliac arch was removed, exposing the dura mater. The dural membrane was pierced with a 23G needle, releasing clear CSF. A polyethylene catheter (O.D. 0.6 mm, I.D. 0.2 mm; 20 cm in length) pre-filled with saline, was carefully advanced a distance of 1 cm into the intrathecal space and a small volume of saline (20 μL) was administered through the 15 catheter. If leakage of saline around the catheter was observed, the rat was excluded from further experimentation. After successful completion of the 'leak test', the intrathecal (i.t.) catheter was fixed with dental cement onto the surrounding muscle ~ 2 cm from L5, exteriorised through a subcutaneous (s.c.) tunnel to a small incision at the base of the neck and sutured in position. After suturing of the lumbar muscles and skin, rats received benzylpenicillin (50000 IU i.p.) and enrofloxacin (5 mg•kg-1 s.c.) to prevent infection and were kept warm during recovery from anaesthesia. Following completion of the surgery, rats were housed singly for a recovery period of 3-4 days prior to i.t. drug administration. On the day following surgery, the local anaesthetic, lignocaine (2%, 20 μL) was administered via the i.t. catheter. If complete paralysis of both hind legs was not observed, rats were excluded from further experimentation.

Drugs Administered
MrIA was prepared in 5 mM sodium acetate buffer at pH 5.5 at delivered to rats in a single bolus dose of 0.2–30 nmoles. Stock solutions of the peptides were quantified relative to an amino acid analysed stock solution by reversed phase HPLC with u.v. detection at Xenome Ltd. Morphine HCl powder was purchased from the Royal Brisbane Hospital Central Pharmacy (Herston, Queensland) and dissolved in normal saline to prepare a stock concentration of 10 μg/10 μl (morphine base). Each rat received 3.5–50 nmol (10-15 μl) of morphine. All dilutions were made with normal saline. All i.t. injections were followed by a saline flush (20 μL) to ensure complete peptide delivery into the intrathecal space.

Storage of Stock Solutions
Aliquots (10 μL) of stock solutions were stored at –20°C prior to use for animal experimentation. Immediately prior to experimentation, aliquots of the relevant compound 10 were thawed at room temperature and then diluted to the required concentration with sterile saline to achieve the desired final concentration for subsequent i.t.. Unused portions were discarded to waste to ensure that compounds only underwent one freeze-thaw cycle.

Intrathecal Drug Dosing
On day 14 post-CCI surgery, individual groups of drug-naïve-CCI rats received an i.t. bolus injection of MrIA, morphine or saline in a volume of 10–15 μL. Antinociception was assessed using von Frey filaments until responses returned to baseline.

Assessment of antinociception: CCI rats using von Frey filaments . Tactile allodynia, the distinguishing feature of neuropathic pain, was quantified using von Frey filaments which were used to apply a non-noxious mechanical stimulus (light pressure) to the hindpaw. Rats were transferred to wire mesh testing cages (20 cm x 20 cm x 20 cm) and allowed to acclimatize for 10 min. Von Frey filaments were used to determine the lowest mechanical threshold required for a brisk paw withdrawal reflex. Briefly, starting with the von Frey filament that produced the lowest force, the filament was applied to the plantar surface of the hindpaw until the filament

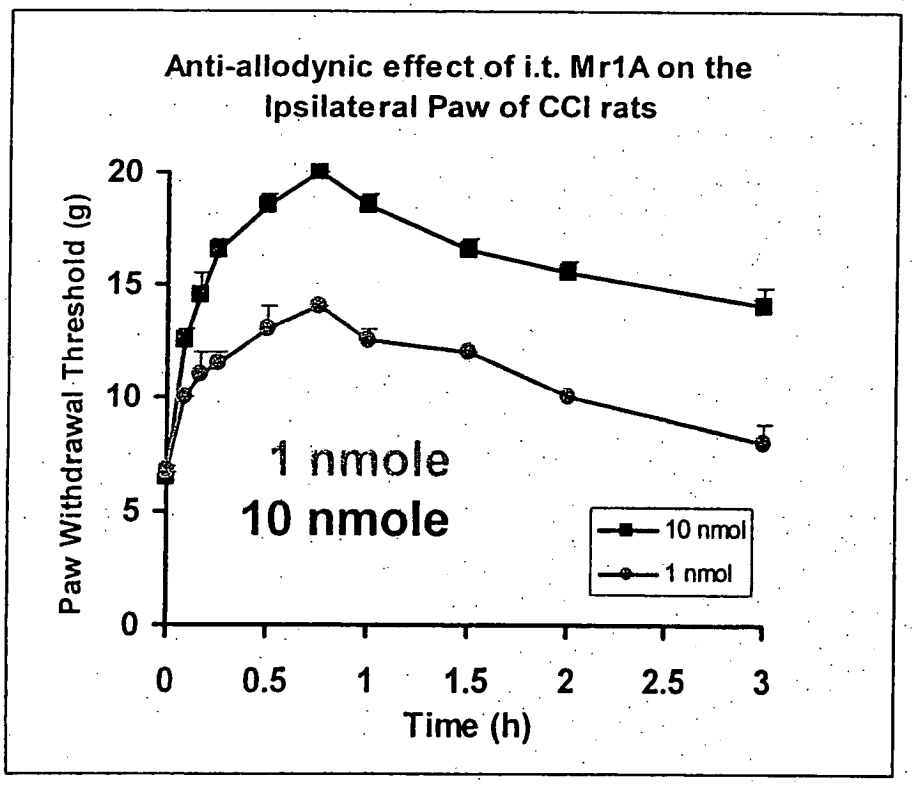
buckled slightly. Absence of a response after 5 s prompted use of the next filament of increasing weight. Filaments used produced a buckling weight of 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 g and these were calibrated regularly. A score of 20 g was given to animals that did not respond to any of the von Frey filaments. Paw withdrawal thresholds (g) were converted to area under the curve (AUCh). The maximum response on the ipsilateral side was 45 AUCh

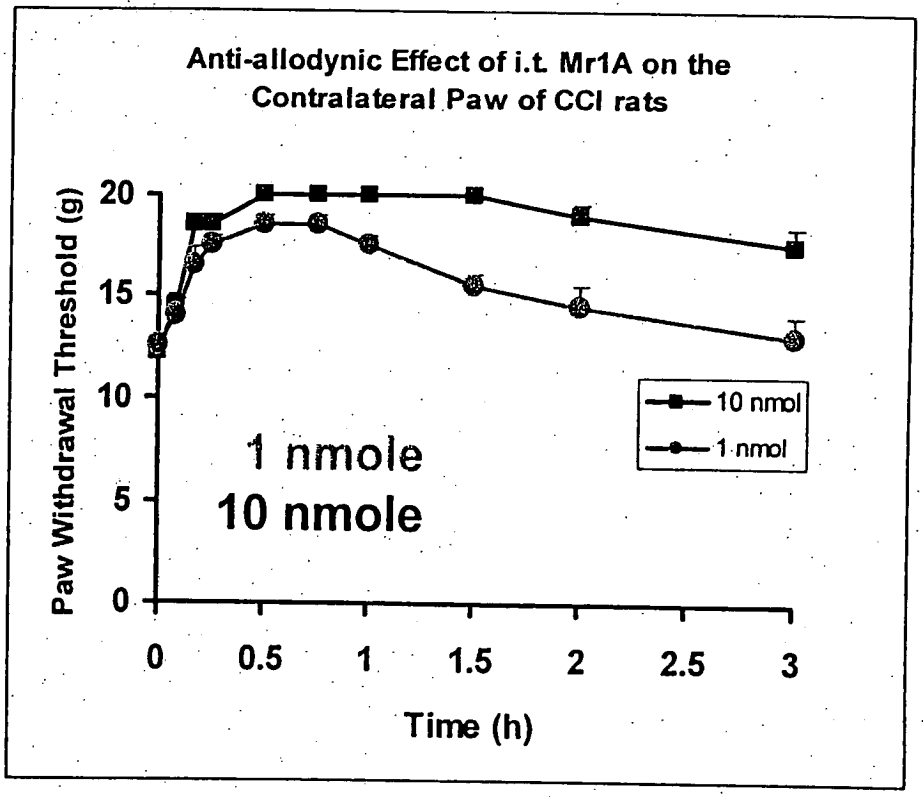
Verification of correct i.t. catheter placement
At the completion of each experiment, malachite green dye (30 μL) was injected via the i.t. catheter whilst rats were lightly anaesthetised with O_2:CO_2 (50%:50%). Thirty seconds later, rats were decapitated and the spinal column was exposed surgically. Data from rats where there was evidence of subcutaneous dye leakage at the site where the catheter entered the back muscles above L6 or failure of the dye to distribute at least 3-4 cm along the spinal cord, were excluded from the analysis.

Data Analysis
The areas under the degree of antinociception versus time curves (AUC values) for each of the peptides were calculated from time = 0 to 3 h. Dose-response curves for each of the peptides were constructed by plotting AUC values versus the i.t. peptide dose (expressed in 15 nmol per rat).

Results
Both MrIA (Figure 1) and morphine (data not shown, reference) produced antinociceptive effects in a rat model of neuropathic pain when injected as a single bolus dose by the intrathecal route (i.t.). These effects were dose-dependent. MrIA at close to a maximum efficacious dose produced antinociceptive effects that lasted at least as long as morphine.

i.t. χ-MrIA produces antiallodynia





xenome